Tautomerism of Xanthine Oxidase Substrates Hypoxanthine and Allopurinol

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The tautomerism of neutral hypoxanthine and allopurinol in the gas phase and in aqueous solution has been examined by theoretical methods. The tautomeric equilibrium in the gas phase was studied from semiempirical and *ab initio* quantum mechanics (QM) and also from density functional theory (DFT) calculations. Electron correlation effects were included in *ab initio* computations at the Møller−Plesset level, and DFT calculations were carried out using the Becke3−Lee−Yang−Parr functional. The influence of the solvent was examined from self-consistent reaction field calculations performed with different continuum models. The results provide a detailed picture of the tautomerism of these biologically relevant compounds. Comparison with available experimental data provides support for the quality of results derived from theoretical computations. Inspection of the most stable tautomeric forms allows discussion of the functional implications of tautomerism for recognition and binding of these molecules to xanthine oxidase.

Introduction

Tautomerism determines the specific pattern of hydrogen-bond donors and acceptors available to a specific molecule. It determines the ability of the molecule to establish interactions with other compounds. Indeed, the structural differences between tautomers also modulate the reactive pathways of the molecule for attack of nucleophilic and electrophilic reagents. The equilibrium between tautomers is largely influenced by the attachment of particular substituents to the "core" of the molecule, and the marked role played by the solvent in determining the relative population of tautomers has also been recognized.[1] The susceptibility of the tautomeric equilibrium to the environment, i.e., the presence of substituents, solvation in polar or apolar solvents, or the transfer between different solvents, has been the subject of many studies.[2,3] This tautomeric susceptibility offers a wide range of possibilities in the design of molecules.

The importance of tautomerism for recognition between molecules is crucial in biochemical and pharmacological research. This is clearly illustrated by the intense research effort devoted to the tautomerism of nucleic acid bases.[4] The maintenance of genetic information, which is determined by the hydrogen-bonded pairing between adenine−thymine and guanine−cytosine, ultimately relies on the existence of purine and pyrimidine bases in definitive tautomeric forms. The standard Watson−Crick pairing between the canonical tautomers also provides a specific pattern of hydrogen-bond interactions in both the major and minor grooves,[5] which allows DNA to be read without the opening of the double helix. Indeed, the appearance of mutations in DNA has been related to minor tautomeric forms.[1a,6]

Less attention has been paid to the primordial role of tautomerism in other relevant biological phenomena, like the enzymatic degradation of purine and pyrimidine

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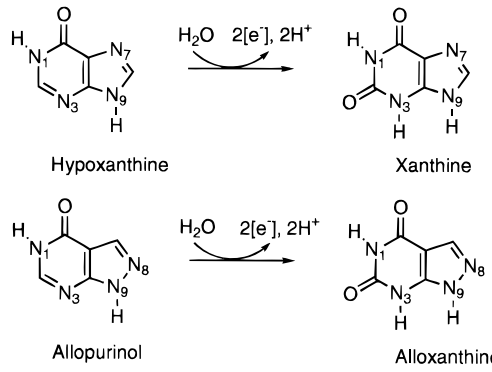

Figure 1. Representation of the enzymatic process catalyzed by xanthine oxidase.

nucleic bases. The importance of tautomerism in the degradation of purine nucleosides by adenosine deaminase (ADA, EC 3.5.4.4) has been demonstrated by our group.[7] ADA is a key enzyme in the catabolism of adenosine that catalyzes its conversion to inosine. This latter nucleoside is subsequently hydrolyzed, yielding the ribose and the purine base, hypoxanthine (1,7-dihydro-6*H*-purin-6-one), which is occasionally found in minor amounts as a constituent of transfer RNA.[8] As an intermediate product in purine catabolism, hypoxanthine is oxidized to xanthine and eventually to uric acid in man. Defects in purine metabolism increase the content of uric acid and lead to the deposition of sodium hydrogen urate monohydrate crystals in joints. This disease, known as gout, is clinically treated by the antihyperuricemic drug allopurinol[9] (pyrazolo[3,4-*d*]pyrimidin-6-one), which has also been used in conjunction with anticancer drugs that impede RNA biosynthesis and as adjunct therapy combined with 6-mercaptopurine in the treatment of leukemia.[10]

Xanthine oxidase (xanthine:O_2 oxidoreductase; EC 1.2.3.2) catalyzes the conversion of hypoxanthine to xanthine (see Figure 1). This enzyme, and the related xanthine dehydrogenase (xanthine:NAD^+ oxidoreductase; EC 1.2.1.37), are composed of two equivalent, independent subunits.[11] Each subunit contains one molybdenum atom, one molecule of flavin adenine dinucleotide (FAD), and two distinct iron−sulfur clusters. A pterin cofactor, which seems to coordinate with the molybdenum *via* its dithiolene side chain, may modulate the reactivity of the molybdenum center and/or its reduction potential.[12] In addition, a phosphoserine residue seems to be present at the active site, but a specific role for this group remains to be established. The enzyme contains two spatially separated substrate-binding sites communicated by an internal electron transport chain, which transfers electrons from the reducing substrate to the oxidizing agent. The enzyme follows a two-site ping-pong mechanism,[11c,13] in which all the prosthetic groups participate in the electron transport chain.

This topographical arrangement permits two different strategies in the design of inhibitors of xanthine oxidase, i.e., inhibitors acting at the purine binding site or in the electron transport chain. Thus, benzimidazole derivatives have been found to be tight binding inhibitors at the FAD cofactor site.[14] Purine-like inhibitors acting at the hydroxypurine site, like allopurinol, have also been designed.[15] However, they can have the undesirable effect of interfering with other aspects of purine metabolism.[16] Knowledge of the molecular interactions that modulate the binding to the purine site would be very helpful in the design of potent, specific inhibitors lacking secondary effects.

Allopurinol closely resembles hypoxanthine (Figure 1), since they differ only in the position of the nitrogens in the five-membered ring. Accordingly, a common pattern of potential interactions is expected upon anchoring of the two molecules at the purine active site. This would be modulated by the tautomerism at the imidazole and pyrazole rings, in addition to the lactam−lactim equilibrium at the six-membered ring. In this study, we examine the tautomerism of hypoxanthine and allopurinol in the gas phase and in aqueous solution. *Ab initio* quantum mechanical methods at the SCF and Møller−Plesset levels and density functional calculations are used to explore the tautomeric preference in the gas phase. The solvent effect is introduced by self-consistent reaction field continuum methods. Inspection of the tautomeric preference allows discussion of the functional implications of tautomerism in the recognition of these substrates by xanthine oxidase.

Methods

The study of all the tautomers of hypoxanthine and allopurinol at a high *ab initio* quantum mechanical (QM) level is exceedingly expensive. Accordingly, a stepwise elimination scheme was used, in which the relative stability between all the tautomers was determined at a low level of QM theory, and only the most stable species were further analyzed at the highest level. Thus, the stability in the gas phase was determined at the AM1[17] semiempirical level, and the influence of hydration on the tautomerism was estimated from AM1-SCRF calculations (see below). Those tautomers whose stability was less than 15 kcal/mol above that of the most stable species (either in the gas phase or in aqueous solution) were considered for further analysis at the *ab initio* level. Initially, gas phase geometry optimization at the HF/6-31G(d)[18] level was performed. In all cases the nature of the minimum energy structure was verified by frequency analysis. The solvent effect was estimated by *ab initio* 6-31G(d) SCRF calculations (see below). Typically, those tautomers whose free energy difference was less than 10 kcal/mol above that of the most stable species (either in the gas phase or in aqueous solution) were considered in the final part of the study.

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In the final study single point calculations at the SCF and MP2[19] levels were carried out with the 6-31+G(d,p) basis.[20] In addition, density functional calculations (DFT) were performed with the Becke3−Lee−Yang−Parr (B3LYP) functional.[21] The HF/6-31G(d) optimized geometry was used in all these calculations. Previous studies have shown that the refinement of structural parameters at higher computational levels has little effect on the tautomerism of closely related structures.[4m] In all cases, zero-point energies and thermal and entropic corrections were computed from the HF/6-31G(d) geometries in the rigid rotor−harmonic oscillator approximation using the standard procedure in Gaussian 92-DFT.[22] A temperature of 298 K was considered for the calculation of thermal and entropic corrections.

The free energy of tautomerization in aqueous solution was determined according to eq 1. Relative free energies of hydration ($\Delta\Delta G_{A\rightarrow B}^{aq}$) were computed from the absolute free energies of hydration (ΔG_A^{hyd}, ΔG_B^{hyd}) as determined from SCRF calculations performed with the AM1[23] and *ab initio* 6-31G(d)[24] optimized versions of the continuum model developed by Miertus, Scrocco, and Tomasi (MST).[25] The corresponding gas phase optimized geometries were used in calculations, since small geometrical effects are expected for rigid molecules like those considered here.[7] Nevertheless, in order to assess the relevance of solvent-induced geometrical changes on the stability of tautomers, AM1−SM2 calculations[26] were performed using the gas phase AM1 geometry and by optimizing the geometry in aqueous solution.

$$\Delta G_{A\rightarrow B}^{aq} = \Delta G_{A\rightarrow B}^{gas} + \Delta G_B^{hyd} - \Delta G_A^{hyd} = \Delta G_{A\rightarrow B}^{gas} + \Delta\Delta G_{A\rightarrow B}^{hyd} \quad (1)$$

Gas phase calculations were carried out using the MOPAC93-Rel.A[27] and Gaussian 92-DFT[22a] computer programs. MST calculations were performed with locally modified versions of MOPAC93-Rel A and MonsterGauss.[28] AM1-SM2 calculations were performed using the AMSOL program[29] developed by Cramer and Truhlar. All simulations were performed on the Cray-YMP of the Centre de Supercomputació de Catalunya and on HP and SGI workstations in our laboratory.

Results

Tautomerism of Hypoxanthine and Allopurinol in the Gas Phase. Seven tautomers of hypoxanthine were considered in the final study after screening[30] in the stepwise elimination protocol (see Methods): four keto (H19, H17, H39, and H37) and three enol (H96t, H96c, and H76c) forms, which are shown in Figure 2. At the MP2/6-31+G(d,p) level (method C in Table 1), the keto tautomer H17 is the most stable tautomer in the gas phase. The difference in stability due to proton tautomerism between nitrogens N7 (H17) and N9 (H19) is small (less than 1 kcal/mol). Tautomerism between six-membered ring nitrogens, N1 and N3, clearly favors the N1−H form, since the relative free energy difference

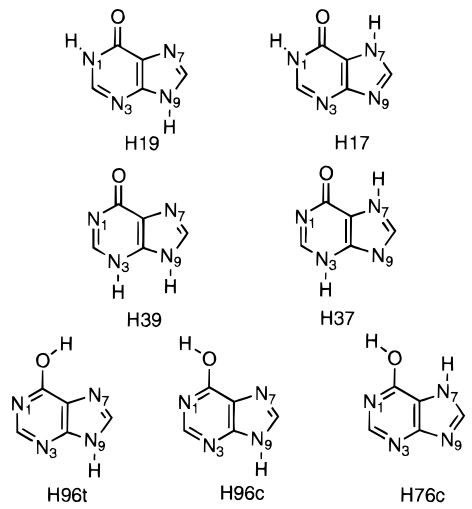

Figure 2. Representation of the seven tautomeric forms of neutral hypoxanthine included in the final study after the stepwise elimination process (see text for details).

Table 1. Differences in Energy, Enthalpy and Free Energy in the Gas Phase (Relative to the Tautomer H19) for Selected Tautomers of Hypoxanthine Determined at the Computational Levels: (A) HF/6-31G(d)//HF/6-31G(d); (B) HF/6-31+G(d,p)//HF/6-31G(d); (C) MP2/6-31+G(d,p)//HF/6-31G(d); (D) B3LYP(6-31+G(d,p))//HF/6-31G(d)[a]

tautomer	method	ΔE	ΔH	ΔG
H17	A	0.1	0.2	0.1
	B	−0.4	−0.4	−0.4
	C	−0.9	−0.9	−0.9
	D	−0.8	−0.8	−0.8
H39	A	21.4	20.7	20.1
	B	20.8	20.2	19.5
	C	21.2	20.5	19.9
	D	20.2	19.6	18.9
H37	A	8.5	8.3	8.1
	B	7.6	7.5	7.2
	C	7.2	7.1	6.9
	D	6.7	6.6	6.3
H96t	A	5.1	4.9	5.0
	B	3.2	3.0	3.1
	C	3.1	2.8	2.9
	D	4.3	4.0	4.1
H96c	A	3.5	3.3	3.4
	B	1.6	1.4	1.5
	C	1.9	1.6	1.7
	D	2.8	2.6	2.7
H76c	A	7.2	6.8	6.8
	B	5.1	4.7	4.7
	C	4.2	3.8	3.8
	D	5.3	4.9	4.9

[a] The nomenclature for the tautomers is given in Figure 2. All values are in kcal/mol.

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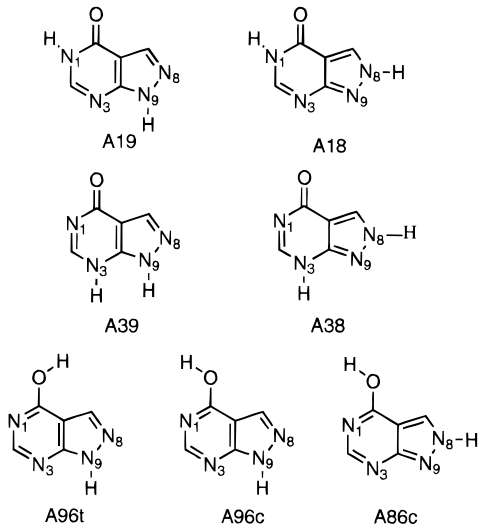

Figure 3. Representation of the seven tautomeric forms of neutral allopurinol included in the final study after the stepwise elimination process (see text for details).

between tautomers H17 and H37 amounts to around 8 kcal/mol. The preference for the N1−H tautomer is even greater when the H19 and H39 forms are considered. This is probably due to increased repulsion between the hydrogen atoms bound to N3 and N9 in this latter form. The stability of the three enol species relative to the tautomer H17 ranges from 2.6 to 4.7 kcal/mol, revealing that they are more stable than the N3−H keto forms.

Seven tautomers of allopurinol (Figure 3) were also selected after the stepwise elimination protocol: four keto (A19, A18, A39, and A38) and three enol (A96t, A96c, and A86c) forms. The free energy differences relative to the tautomer A19 at the different levels of theory for selected tautomers are given in Table 2. The preferred species in the gas phase is the keto tautomer A19. At the MP2/6-31+G(d,p)//HF/6-31G(d) level the tautomer A18 is destabilized by 3.0 kcal/mol. As was found for hypoxanthine, the two N3−H keto tautomers are less stable than the N1−H species by at least 9 kcal/mol, the tautomer A38 being around 5 kcal/mol more stable than the form A39. Again, the enol species are preferred over the N3−H keto tautomers, but they are largely disfavored in the gas phase. Thus, A96c is less stable than A19 by 4.2 kcal/mol at the MP2/6-31+G(d,p)//HF/6-31G(d) level, whereas the species A96t and A86c are destabilized by around 10 kcal/mol.

It is interesting to compare the different theoretical estimates reported in Tables 1 and 2. The size of these molecules prevents high-level *ab initio* calculations to be performed, and confidence in the results must be gained from the convergence achieved as the computational level is increased. Furthermore, this comparison may indicate alternative less expensive methods of calculation than the MP2/6-31+G(d,p) one. Comparison of the results shows a close agreement between relative stabilities determined at the SCF-RHF and MP2 levels when the 6-31+G(d,p) basis set is used. This agreement extends, in general, to calculations at the HF/6-31G(d) level, giving confidence in the stepwise elimination protocol adopted in this study. Finally, small differences are found between the results determined from calculations at the MP2 and DFT levels, which supports the relative stabilities of tautomers.

Table 2. Differences in Energy, Enthalpy and Free Energy in the Gas Phase (Relative to the Tautomer A19) for Selected Tautomers of Allopurinol (see Table 1 for Computational Methods)[a]

tautomer	method	ΔE	ΔH	ΔG
A18	A	4.9	4.9	4.9
	B	4.7	4.7	4.8
	C	3.0	3.0	3.0
	D	3.6	3.6	3.7
A39	A	19.0	18.4	17.8
	B	18.5	17.9	17.9
	C	18.9	18.4	17.8
	D	18.1	17.5	17.0
A38	A	14.2	13.9	13.7
	B	13.7	13.4	13.1
	C	13.2	13.0	12.7
	D	12.6	12.3	12.1
A96t	A	13.0	12.5	12.5
	B	11.3	10.8	10.8
	C	10.3	9.8	9.8
	D	11.1	10.7	10.6
A96c	A	6.3	6.0	6.1
	B	4.6	4.3	4.4
	C	4.4	4.1	4.2
	D	5.1	4.8	4.9
A86c	A	16.7	16.5	16.7
	B	14.8	14.6	14.8
	C	10.4	10.2	10.4
	D	12.4	12.2	12.4

[a] The nomenclature for the tautomers is given in Figure 3. All values are in kcal/mol.

Table 3. Differences in the Free Energies of Hydration (Relative to the Tautomer H19) of Selected Tautomers of Hypoxanthine[a]

tautomer	AMSOL (AM1−SM2,g)	AMSOL (AM1−SM2,s)	MST (AM1)	MST (6-31G(d))
H17	1.6	1.7	1.0	0.7
H39	−5.3	−6.7	−8.4	−9.6
H37	−1.6	−2.3	−1.2	−1.7
H96t	3.3	4.0	4.9	5.2
H96c	2.9	3.5	4.2	5.2
H76c	2.7	3.1	2.7	3.6

[a] AMSOL (AM1−SM2) calculations were performed with the geometry optimized in the gas phase (AMSOL (AM1−SM2,g)) or with geometry optimization in solution (AMSOL (AM1−SM2,s)). MST calculations were performed at the AM1 (MST (AM1)) and 6-31G(d) (MST (6-31G(d))) levels using the corresponding gas phase optimized geometry. All values are in kcal/mol.

Tautomerism of Hypoxanthine and Allopurinol in Aqueous Solution. The solvent effect on tautomerism was examined by SCRF semiempirical and *ab initio* calculations with a 2-fold purpose. First, the influence of solvent-induced geometry changes on the relative stability of tautomers was investigated from AM1−SM2[26] calculations. Second, the magnitude of the solvent effect on the relative stability was examined using two different continuum models: the generalized Born model as implemented in AM1−SM2 and the MST[23,24] version of the polarizable continuum method.[25] MST calculations were performed at the semiempirical AM1 and *ab initio* HF/6-31G(d) levels. Comparison of the corresponding free energies of hydration allowed us to assess the reliability of the solvent-induced changes in the relative stability of tautomers.

The differences in the free energy of hydration ($\Delta\Delta G_{A\rightarrow B}^{hyd}$) determined from AMSOL AM1−SM2 and MST continuum calculations for selected tautomers of hypoxanthine and allopurinol are given in Tables 3 and

Table 4. Differences in the Free Energies of Hydration (Relative to the Tautomer A19) of Selected Tautomers of Allopurinol (see Table 3 for Computational Methods)[a]

tautomer	AMSOL (AM1−SM2,g)	AMSOL (AM1−SM2,s)	MST (AM1)	MST (6-31G(d))
A18	−1.8	−2.0	−3.2	−2.6
A39	−5.5	−6.8	−7.5	−8.0
A38	−4.9	−5.7	−5.7	−5.2
A96t	3.4	4.1	1.9	1.3
A96c	3.6	4.1	3.8	4.4
A86c	−1.3	−2.0	−1.6	0.1

[a] All values are in kcal/mol.

4. The relative free energies of hydration determined from AMSOL calculations using the gas phase geometry or upon geometry optimization in aqueous solution are very similar. The largest variation, which amounts to 1.4 kcal/mol, is found for the tautomer H39. However, this change is not relevant, since the free energy difference of this tautomer with respect to the most stable species (H17) in the gas phase is much larger (around 21 kcal/mol). The root mean square deviation in relative free energies of hydration upon geometry optimization amounts to 0.6 kcal/mol for the rest of the tautomers. This suggests that changes in structural parameters do not significantly modify the relative free energy of hydration of the tautomers and supports the use of gas phase geometries in MST calculations.

The results given by AM1−SM2 and MST calculations show good agreement. Despite some differences in the relative free energies of hydration, a notable qualitative agreement is found between the results determined from AM1−SM2 and MST (semiempirical and *ab initio*) calculations, which is even more remarkable bearing in mind the different functional forms of these two SCRF continuum models. All the methods indicate a preferential hydration of the N3-H keto tautomers (H39, H37, A39, and A38) over the N1−H keto species (H19, H17, A19, and A18). For hypoxanthine, the N9−H tautomers H39 and H19 are better hydrated than the corresponding N7−H forms (H37 and H17). Such a preference is 4 kcal/mol or even larger for the N3−H tautomers and varies between 0.7 (MST/6-31G(d)) and 1.7 (AM1−SM2) kcal/mol for the N1−H species. Different behavior is found for allopurinol. The tautomer A39 is also better hydrated than the A38. However, the solvent-induced stabilization of the tautomer A18 is larger (around 2−3 kcal/mol) than that estimated for A19. Finally, all the enol forms, with the exception of the species A86c, are greatly destabilized upon solvation.

The relative free energies of hydration determined from AMSOL and MST calculations (Tables 3 and 4) were subsequently used to estimate the differences in the free energy of tautomerization in aqueous solution ($\Delta G_{A\rightarrow B}^{aq}$) according to eq 1. The free energy of tautomerization in the gas phase ($\Delta G_{A\rightarrow B}^{gas}$) was taken from the value computed at the MP2/6-31+G(d,p)//HF-6-31G(d) level (Tables 1 and 2). Values of $\Delta G_{A\rightarrow B}^{aq}$ for tautomers of hypoxanthine and allopurinol are reported in Tables 5 and 6, respectively.

The relevance of the solvent effect on tautomerism of hypoxanthine is apparent from comparison of the results in Tables 1 and 5. The tautomer H17, the most stable species in the gas phase, is destabilized with regard to H19 upon solvation. Depending on the method used to compute the relative free energy of hydration, the stability of H17 is similar to (−0.2 kcal/mol according to the MST(6-31G(d)) method) or lower than (0.7 kcal/mol according to AM1−SM2) the value estimated for H19. Despite the large stabilization of the two N3−H keto tautomers induced upon solvation (Table 3), these species are less stable than the N1−H keto forms by more than 5 kcal/mol. Indeed, because of the solvent-induced destabilization, the enol forms are less favored than in the gas phase, the free energy difference being at least 4.6 kcal/mol with respect to the H19 species.

Table 5. Free Energies of Tautomerization ($\Delta G_{A\rightarrow B}^{aq}$; Relative to the Tautomer H19) of Selected Tautomers of Hypoxanthine[a]

tautomer	$\Delta G_{A\rightarrow B}^{aq-AMSOL}$	$\Delta G_{A\rightarrow B}^{aq-MST(AM1)}$	$\Delta G_{A\rightarrow B}^{aq-MST(6-31G(d))}$
H17	0.7	0.1	−0.2
H39	14.6	11.5	10.3
H37	5.3	5.7	5.2
H96t	6.2	7.9	8.1
H96c	4.6	5.9	6.9
H76c	6.5	6.5	7.4

[a] The values were estimated from the addition of the free energy of tautomerization in the gas phase at the MP2/6-31+G(d,p))//HF/6-31G(d) level to the free energy of hydration determined from AMSOL (AM1-SM2), MST (AM1), and MST (6-31G(d)) calculations (eq 1). All values are in kcal/mol.

Table 6. Free Energies of Tautomerization ($\Delta G_{A\rightarrow B}^{aq}$; Relative to the Tautomer A19) of Selected Tautomers of Allopurinol (see Table 5 for Computational Methods)[a]

tautomer	$\Delta G_{A\rightarrow B}^{aq-AMSOL}$	$\Delta G_{A\rightarrow B}^{aq-MST(AM1)}$	$\Delta G_{A\rightarrow B}^{aq-MST(6-31G(d))}$
A18	1.2	−0.3	0.5
A39	12.4	10.3	9.8
A38	7.8	5.2	7.5
A96t	13.2	11.7	11.1
A96c	7.8	8.0	8.6
A86c	9.1	8.8	10.5

[a] All the values are in kcal/mol.

The free energies of tautomerization in aqueous solution for tautomers of allopurinol are reported in Table 6. The solvent also has a great influence on the relative stabilities of tautomers. The tautomer A18, which is less stable than the A19 in the gas phase by 3.0 kcal/mol at the MP2/6-31+G(d,p)//HF-6-31G(d) level, is clearly stabilized upon solvation. The free energy difference with respect to the species A19 ranges from −0.3 kcal/mol to 1.2 kcal/mol, when the MST(AM1) and AM1−SM2 values are considered. Despite the large solvent-induced stabilization of the N3−H keto tautomers, they are still disfavored by 5 kcal/mol or more. Finally, the enol forms are disfavored in aqueous solvent by at least 8 kcal/mol.

Discussion

High-level QM *ab initio* calculations and SCRF continuum methods provide a detailed picture of the tautomerism of hypoxanthine and allopurinol in the gas phase and in aqueous solution. Analysis of the relative stabilities of the different tautomeric species can be used to elucidate the reactive characteristics of these two molecules. In particular, inspection of the potential interactions of the preferred tautomers with hydrogen-bond donors and acceptors can be used to gain insight into their recognition pattern at the active site of xanthine oxidase.

The reliability of the results for hypoxanthine and allopurinol in the gas phase is supported by the agreement found between SCF and MP2 values when a large, flexible basis set is used. The similarity with the results determined within the nonlocal DFT formalism gives further confidence. The influence of higher order correlation effects on the tautomeric preference in the gas

phase determined at the MP2/6-31+G(d,p)//HF/6-31G(d) level for hypoxanthine and allopurinol is expected to be small, as suggested by the results reported in recent studies. Thus, a negligible effect is expected for the proton tautomerism between nitrogens according to the MP2 and MP4 results reported for tautomers of imidazole[3d] and 7-aminopyrazolopyrimidine.[7c] Indeed, a small difference is found between the MP2 and MP4 results determined with the 6-311++G(d,p) basis for the relative stability of keto and enol forms of guanine and cytosine (see ref 7m and references therein). A similar finding is observed for the tautomerism of 3-hydroxypyrazole involving keto and enol forms, since extension from MP2 to MP4 or coupled-cluster single- and double-excitation calculations has a small effect on the relative energies.[3e] Finally, treatment of electron correlation using the second-order perturbation theory has a relevant effect on the relative energies between the oxo and hydroxy forms of 5-hydroxyisoxazoles,[3b] much larger than the effect due to higher order effects at either MP4 or coupled-cluster levels of theory. On the other hand, despite some numerical uncertainties in the values of the relative free energies of hydration, the agreement found between values derived from AM1−SM2 and MST calculations is good. This gives confidence in the theoretical estimates of the solvent influence on the relative stabilities of the tautomers in aqueous solution.

The present results indicate that hypoxanthine can be found in the gas phase as a mixture of two predominant tautomeric forms: the N1−H keto species H17 and H19. According to our best estimate, the tautomer H17 is 0.9 kcal/mol more stable than H19. This value is in reasonable agreement with previous semiempirical results determined at the CNDO/2 level.[31] However, this finding is in contrast with recent HF calculations[32] performed with the 6-31G(d,p) and MIDI basis sets, which indicate that the H19 species has a similar stability or is even more stable than the H17 tautomer. It is worth noting that our HF/6-31G(d) results also indicate a similar stability between the two tautomers, but extension of the basis set and inclusion of correlation effects results in the H17 form are preferred in the gas phase.

The population of N3−H keto tautomers is negligible, since they are largely destabilized by at least 8 kcal/mol with respect to the species H17. However, a small fraction of enol forms cannot be ruled out, since the tautomer H96c is 2.6 kcal/mol less favored than H17. According to the relative stabilities determined at the MP2/6-31+G(d,p) level for the species H17, H19, and H96c, the population of these tautomers in the gas phase is 81%, 18%, and 1%, respectively. This agrees with experimental evidence gained from ultraviolet photoelectron spectra, which clearly indicates that the tautomer H17 is more stable than H19 in the gas phase.[33]

Allopurinol can be found in the gas phase mainly in the tautomeric form A19. At the MP2/6-31+G(d,p) level the free energy difference with respect to the next most stable tautomer (A18) is 3 kcal/mol, which is somewhat lower than the estimate of 5.4 kcal/mol derived from HF/MIDI calculations.[32] The large difference in stability (>12 kcal/mol) precludes N3−H keto tautomers. Indeed, the presence of enol forms is ruled out, except in the case of A96c, whose relative stability is 4.2 kcal/mol. From these values the population in the gas phase of tautomers A19, A18, and A96c is predicted to be 99.3%, 0.6%, and 0.1%, respectively. Unfortunately, to our knowledge there are no experimental data available for comparison, although the estimated free energy differences are large enough to guarantee the predominance of tautomer A19.

Solvation greatly modulates tautomerism of hypoxanthine. The most relevant effect is the solvent-induced stabilization of H19, which varies from −0.7 to −1.7 kcal/mol depending on the method used to estimate the free energy of hydration. This suggests a change from the gas phase, since the stability of the two N1−H keto tautomers becomes more similar upon solvation. In fact, our estimates of the free energy difference between these tautomers range from −0.2 to +0.7 kcal/mol. The N3−H keto tautomers are stabilized upon solvation, but they are still disfavored by more than 5 kcal/mol with respect to the H19 form. Moreover, the enol tautomers are destabilized by at least 4.6 kcal/mol. Therefore, only the N1−H keto tautomers (H19 and H17) are expected in aqueous solution. A precise determination of the tautomer population is not feasible because of the range of error in the calculations, but there must be significant fractions of both H19 and H17. According to the present results, the population of tautomer H19 ranges between 42% and 77%.

Experimental evidence clearly indicates that hypoxanthine exists as the N1-H keto species in aqueous[34] and dimethyl sulfoxide[35] solutions, and this is also the structure determined crystallographically.[36] Unfortunately, experimental data on the tautomerism at the imidazole ring are less conclusive. Thus, ultraviolet spectra in aqueous solution suggest that H19 is predominant, but information gathered from NMR data is equivocal.[34c] Protonation of hypoxanthine occurs at the imidazole ring,[34d,37] and a recent study seems to suggest that protonation probably occurs at N7,[38] which indicates that H19 is the predominant tautomer. ^{13}C-NMR data in dimethylsulphoxide solution indicates that the population of H17 is around 58%.[35] The crystal structure of the compound isolated corresponds to the form H19,[36b] each molecule being connected by six hydrogen-bonds to neighboring molecules. Crystallographic studies of metal-chelating hypoxanthine complexes have elucidated three different coordination types.[39] The first involves monomeric complexes with monodentate coordination to N7, suggesting that the H19 tautomer predominates over the H17 form. The second involves dimeric complexes in which N3 and N9 act as chelating positions, suggesting that H17 is more stable than H19. Finally, the third type appears in polymeric complexes, where hypoxanthine

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coordinates through N3 and N7 to different metal ions, suggesting now that H19 is more stable than H17. No coordination through the N1−H tautomers are more stable than the N3−H. All this experimental evidence clearly suggests that the N1−H tautomers are more stable than those for N3-H. Furthermore, experimental results suggest that the prototropic tautomerism at the imidazole ring for N1−H keto tautomers is extremely sensitive to the environment and that the stability of tautomers H17 and H19 in polar environments are unlikely to be very different.

As was found for hypoxanthine, both the enol and N3−H keto tautomers of allopurinol are clearly destabilized with regard to the N1−H keto forms upon solvation, and their populations in aqueous solution are expected to be negligible. Nevertheless, the solvent has a marked influence on the relative stability of A19 and A18, since the latter is better hydrated. Thus, the stability difference between the two tautomers ranges from −0.3 to +1.2 kcal/mol upon solvation, which implies a change in the tautomeric population with respect to the gas phase situation, where A19 was the preferred, if not the only, form. According to our best estimates, the population of tautomer A19 ranges between 38% and 88% in aqueous solution. As noted before for hypoxanthine, the present results do not allow an accurate estimate of the most stable tautomer, but they indicate that the two tautomers probably coexist in aqueous solution.

Both ultraviolet spectra and ^{13}C-NMR data demonstrate that allopurinol exists as a mixture of tautomers A19 and A18 in aqueous solution.[40] The crystal structure of allopurinol corresponds to the form A19,[41] which establishes a dense network of hydrogen-bond interactions. Monodentate metal coordination of neutral allopurinol through the N8 position is generally found,[42] but metalation with rhodium occurs at the N9 position.[43] Therefore, a subtle influence of the environment on the equilibrium between tautomers A19 and A18 is also apparent from these experimental data, which suggests that the stability of these two tautomers must be similar.

It is known that the oxidation process catalyzed by xanthine oxidase (XO) is carried out in such a way that oxygen in transferred from a catalytically labile site in the enzyme to the substrate. Structural studies indicate that the molybdenum center consists of a MoOS unit, the M=O moiety being common to each of the oxomolybdenum enzymes known up to date.[11] The catalytically labile oxygen is probably that of the M=O group, which is regenerated from a solvent water molecule after being incorporated into the substrate in the catalytic process. Therefore, the position of the substrate susceptible to oxidation must be placed close to the molybdenum center upon binding to the enzyme.

The preceding discussion stresses that only the N1−H keto tautomers of hypoxanthine and allopurinol are significant in either the gas phase or in aqueous solution. All share the same pattern of potential hydrogen-bond interactions around the six-membered ring. The differences lie exclusively in the distribution of hydrogen-bond donor and acceptor groups at the imidazole and pyrazole rings (Figure 4). If both hypoxanthine and allopurinol bind specifically to the same catalytic site in the enzyme, a common pattern of interactions for the "bioactive" species is expected, since substrate recognition is modulated by geometric and electrostatic complementarity with the residues of the binding site. Indeed, it is reasonable to assume desolvation of substrate upon binding, and so interactions with groups at the catalytic center properly position the substrate for enzymatic reaction at a given site (oxidation of hypoxanthine and allopurinol occurs at the carbon C2; see Figure 1).

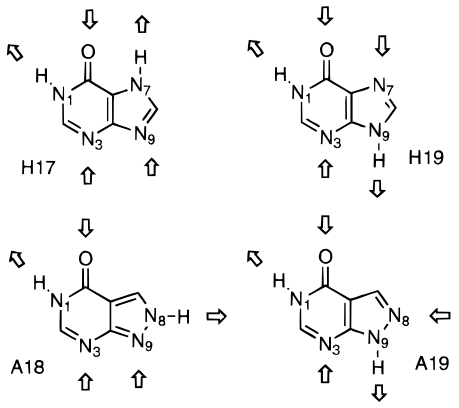

Figure 4. Scheme of the potential hydrogen-bond interactions for the most stable tautomers of hypoxanthine (H17 and H19) and allopurinol (A18 and A19).

The present results show that the equilibrium between the N1−H keto tautomers is altered upon desolvation. The biological importance of desolvation in the mechanism of binding to xanthine oxidase is unclear, since the polarity of the active site of the enzyme is unknown. A notable contribution can be expected due to the presence of molybdenum, but the polarity of the active site is probably lower than that of water. The lack of details on this prohibits a quantitative interpretation of the results obtained here for gas phase and water.

The equilibrium between tautomers A19 and A18 of allopurinol in solution is clearly shifted toward the form A19 in the gas phase, whose population amounts to around 99%. For hypoxanthine, our best estimates of the relative stability in the gas phase indicate that tautomer H17 (81%) is predominant, but tautomer H19 exists in a significant fraction (18%), this latter form probably being the main species in solution. Therefore, tautomers H19 and A19 may be the "bioactive" species at the catalytic site. On this point, comparison of reaction rates of different series of purines by bovine milk xanthine oxidase suggested that attachment of rapidly oxidized substrates may be mediated by interaction of the groupings N3−H,N9 or N3,N9−H.[44] The present results clearly support the latter grouping, since the proton tautomeric equilibrium between N1 and N3 both in the gas phase and in aqueous solution is strongly displaced toward the N1−H species.

Comparison of the corresponding hydrogen-bond sites permits the definition of the basic pattern of interactions with the substrate binding site, which is schematically depicted in Figure 5. This approximated scheme agrees well with experimental evidence available on the sus-

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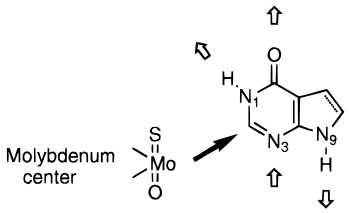

Figure 5. Scheme of the hypothetical recognition pattern at the substrate binding site.

ceptibility of derivatives of hypoxanthine to oxidation by xanthine oxidase.[44] Thus, all mono-N-methylated compounds are not attacked by the enzyme or are oxidized much slower (at least 2 orders of magnitude) than the unmethylated hypoxanthine. This demonstrates the importance of specific H-bond interactions in the recognition process and, consequently, the relevance of tautomerism in the mechanism of enzyme action. The scheme assumes that differences between hypoxanthine and allopurinol at positions 7 and 8 are not relevant for substrate recognition. This is supported by the finding that methylation at position 8 has no significant effect, since the measured enzymatic activity is close to that found for hypoxanthine. Indeed, the refractoriness of 7-methylhypoxanthine[44] can be attributed not to steric hindrance of the methyl group, but rather to the fact that this modification transforms N9 from hydrogen-bond donor (Figure 5) to hydrogen-bond acceptor, which prevents the interaction with the corresponding group at the binding site.

Conclusion

The tautomerism of hypoxanthine and allopurinol exhibits subtle changes depending on the environment. Both in the gas phase and in aqueous solution tautomerism at the six-membered ring is clearly displaced toward the N1−H keto forms. The proton tautomerism at the imidazole or pyrazole ring is more delicate. Our results indicate that a mixture of N7−H and N9−H species is expected in aqueous solution. In the gas phase, nevertheless, allopurinol exists mainly in the N9−H tautomer, whereas for hypoxanthine significant fractions of N9−H and N7−H are expected, even though the latter species predominates.

According to the relative stabilities of tautomers, it is suggested that the "bioactive" species corresponds to the (N1−H, N9−H) keto tautomer. Binding of substrate for oxidation at position C2 is mainly directed by recognition of the two neighboring pyrimidine nitrogens, whose nature allows hydrogen-bond donor (N1−H) and acceptor (N3) interactions with specific groups at the binding pocket. Proper alignment is also provided by anchoring of the carbonylic oxygen (O6) and the imidazole nitrogen N9−H. All these potential interactions may define the recognition pattern between substrate and enzyme. Extension of the present studies to tautomerism of closely related structures may provide an additional basis to test the suitability of this scheme of interactions. The resolution of the 3-D structure of xanthine oxidase in the future will determine the accuracy of the pharmacophoric models presented here.

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